MARKED-UP SPECIFICATION

Please amend the first complete paragraph on page 1 of the specification under the heading Related Application to read as follows:

Related Application

This application is a divisional of U.S. Serial No. 08/960,774, filed October 30, 1997, now issued as U.S. Patent No. 6,239,116B1 on May 29, 2001, which is a continuation-in-part of U.S. Serial No. 08/738,652, filed October 30, 1996, [pending] which is now issued as U.S. Patent No. 6,207,646B1 on March 27, 2001, which is a continuation-in-part of U.S. patent application serial number 08/386,063, filed February 7, 1995 [currently pending] now issued as U.S. Patent No. 6,194,388B1 on February 27, 2001, which is a continuation-in-part of U.S. Patent Application 08/276,358, filed July 15, 1994 which is now abandoned, each of which are incorporated herein by reference in their entirety.

Please rewrite the paragraphs beginning at line 23 on page 9 through line 32 on page 9 as shown.

Figure [A 1]1A. *E. coli* [(l)] (●) and calf thymus DNA [(n)] (■) sequences and LPS (at 10x the concentration of *E. coli* and calf thymus DNA) [(u)] (◆).

Figure 1B. Control phosphodiester oligodeoxynucleotide (ODN) 5' ATGGAAGGTCCAGTGTTCTC 3' (SEQ ID NO:114) [(n)] (■) and two phosphodiester CpG ODN 5' ATCGACCTACGTGCGTTCTC 3' (SEQ ID NO:2) [(u)] (◆) and 5' TCCATAACGTTCCTGATGCT 3' (SEQ ID NO:3) [(l)] (●).

Figure 1C. Control phosphorothioate ODN 5' GCTAGATGTTAGCGT 3' (SEQ ID NO:4) [(n)] (■) and two phosphorothioate CpG ODN 5' GAGAACGTCGACCTTCGAT 3' (SEQ ID NO: 5) [(n)] (■) and 5' GCATGACGTTGAGCT 3' (SEQ ID NO:6) [(l)] (●). Data present the mean ± standard deviation of triplicates.

Please rewrite the paragraph beginning at line 1 on page 10 through line 6 on page 10 as shown.

Figure 2 is a graph plotting IL-6 production induced by CpG DNA in vivo as determined 1-8 hrs after injection. Data represent the mean for duplicate analyses of sera from two mice. BALB/c mice (two mice/group) were inject iv. with 100 µl of PBS [(o)] (□) of 200 µg of CpG phosphorothioate ODN 5' TCCATGACGTTCCTGATGCT 3' (SEQ ID NO:7) [(n)] (■) or non-CpG phosphorothioate 5' TCCATGAGCTTCCTGAGTCT 3' (SEQ ID NO: 8) [(u)] (◆).

Please rewrite the paragraph beginning at line 13 on page 10 through line 22 on page 10 as shown.

Figure 4A is a graph plotting dose-dependent inhibition of CpG-induced IgM production by anti-IL-6. Splenic B-cells from DBA/2 mice were stimulated with CpG ODN 5' TCCAAGACGTTCCTGATGCT 3' (SEQ ID NO:9) in the presence of the indicated concentrations of neutralizing anti-IL-6 [(u)] (◆) or isotype control Ab [(l)] (●) and IgM levels in culture supernatants determined by ELISA. In the absence of CpG ODN, the anti-IL-6 Ab had no effect on IgM secretion [(n)] (■).

Figure 4B is a graph plotting the stimulation index of CpG-induced splenic B cells cultured with anti-IL-6 and CpG S-ODN 5' TCCATGACGTTCCTGATGCT 3' (SEQ ID NO:7) [(u)] (◆) or anti-IL-6 antibody only [(n)] (■). Data present the mean ± standard deviation of triplicates.

were rendered nuclease resistant by phosphorothioate modification of the terminal internucleotide linkages. ODN 1585 (5' GGGGTCAACGTTGAGGGGGG 3' (SEQ ID NO:12)), in which the first two and last five internucleotide linkages are phosphorothioate modified caused an average 25.4 fold increase in mouse spleen cell proliferation compared to an average 3.2 fold increase in proliferation included by ODN 1638, which has the same sequence as ODN 1585 except that the 10 Gs at the two ends are replaced by 10 As. The effect of the G-rich ends is *cis*; addition of an ODN with poly G ends but no CpG motif to cells along with 1638 gave no increased proliferation. For nucleic acid molecules longer than 8 base pairs, non-palindromic motifs containing an unmethylated CpG were found to be more immunostimulatory.

Table 1

ODN	Sequence (5' to 3')†	Stimulation Index' ^{3}H Uridine	Stimulation Index' IgM Production
1 (SEQ ID NO:89)	GCTAGACGTTAGCGT	6.1 ± 0.8	17.9 ± 3.6
1a (SEQ. ID NO:4)	......T.....T.	1.2 ± 0.2	1.7 ± 0.5
1b (SEQ ID NO:13)	......Z.....T.	1.2 ± 0.1	1.8 ± 0.0
1c (SEQ ID NO:14)	.....T.....Z..	10.3 ± 4.4	9.5 ± 1.8
1d (SEQ ID NO:6)	..AT..T...GAGC.	13.0 ± 2.3	18.3 ± 7.5
2 (SEQ ID NO:1)	ATGGAAGGTCCAGCGTTCTC	2.9 ± 0.2	13.6 ± 2.0
2a (SEQ ID NO:15)	..C..CTC..G..T.....	7.7 ± 0.8	24.2 ± 3.2
2b (SEQ ID NO:16)	..Z..CTC.ZG..Z.....	1.6 ± 0.5	2.8 ± 2.2
2c (SEQ ID NO:17)	..Z..CTC..G..T.....	3.1 ± 0.6	7.3 ± 1.4
2d (SEQ ID NO:18)	..C..CTC..G...T..Z..	7.4 ± 1.4	27.7 ± 5.4
2e (SEQ ID NO:19)	.....A..T.....	5.6 ± 2.0	ND
3D (SEQ ID NO:20)	GAGAACGCTGGACCTTCCAT	4.9 ± 0.5	19.9 ± 3.6
3Da(SEQ ID NO:21)	.....T..C.....	6.6 ± 1.5	33.9 ± 6.8
3Db(SEQ ID NO:22)	.....T..C.....G..	10.1 ± 2.8	25.4 ± 0.8
3Dc(SEQ ID NO:23)	...C.A.....	1.0 ± 0.1	1.2 ± 0.5
3Dd(SEQ ID NO:24)	.....Z.....	1.2 ± 0.2	1.0 ± 0.4
3De(SEQ ID NO:25)	.....T.....Z.....	4.4 ± 1.2	18.8 ± 4.4
3Df(SEQ ID NO:26)	.....TA.....	1.6 ± 0.1	7.7 ± 0.4
3Dg(SEQ ID NO:27)	.....T..CC.G.ACTG..	6.1 ± 1.5	18.6 ± 1.5
3M (SEQ ID NO:28)	TCCATGTCGGTCCTGATGCT	4.1 ± 0.2	23.2 ± 4.9
3Ma(SEQ ID NO:29)	......CT.....	0.9 ± 0.1	1.8 ± 0.5
3Mb(SEQ ID NO:30)	.....Z.....	1.3 ± 0.3	1.5 ± 0.6
3Mc(SEQ ID NO:31)	.....T..Z.....	5.4 ± 1.5	8.5 ± 2.6
3Md(SEQ ID NO:7)	......A..T.....	17.2 ± 9.4	ND
3Me(SEQ ID NO:32)	.....T.....C..A.	3.6 ± 0.2	14.2 ± 5.2

4	(SEQ ID NO:90)	TCAACGTT	6.1 ± 1.4	19.2 ± 5.2
4a	(SEQ ID NO:91)	....GC..	1.1 ± 0.2	1.5 ± 1.1
4b	(SEQ ID NO:92)	...GCGC.	4.5.± 0.2	9.6 ± 3.4
4c	(SEQ ID NO:93)	...TCGA.	2.7.± 1.0	ND
4d	(SEQ ID NO:94)	..TT..AA	1.3 ± 0.2	ND
4e	(Residue 2-8 of SEQ ID NO:90; SEQ ID NO: 106)	-......	1.3 ± 0.2	1.1 ± 0.5
4f	(SEQ ID NO:95)	C.....	3.9 ± 1.4	ND
4g	(Residue 11-18 of SEQ ID NO:19; SEQ ID NO:117)	--.....CT	1.4 ± 0.3	ND
4h	(SEQ ID NO:96)	C	1.2 ± 0.2	ND

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' Stimulation indexes are the means and std. dev. derived from at least 3 separate experiments, and are compared to wells cultured with no added ODN. ND = not done. CpG dinucleotides are underlined. Dots indicate identity; dashes indicate deletions. Z = 5 methyl cytosine.

Table 2. Identification of the optimal CpG motif for Murine IL-6 production and B cell activation.

ODN	SEQUENCE (5'-3')		IL-6 (pg/ml)[a]		SI[b]	IgM (ng/ml)[c]
			CH12.LX	SPLENIC B CELL		
512	(SEQ ID No:28)	TCCATGTCGGTCCTGATGCT	300 ± 106	627 ± 43	5.8 ± 0.3	7315 ± 1324
1637	(SEQ ID No:33)	......C..........	136 ± 27	46 ± 6	1.7 ± 0.2	770 ± 72
1615	(SEQ ID No:34)	......G..........	1201 ± 155	850 ± 202	3.7 ± 0.3	3212 ± 617
1614	(SEQ ID No:35)	......A..........	1533 ± 321	1812 ± 103	10.8 ± 0.6	7558 ± 414
1636	(SEQ ID No:36)	.........A.......	1181 ± 76	947 ± 132	5.4 ± 0.4	3983 ± 485
1634	(SEQ ID No:37)	.........C.......	1049 ± 223	1671 ± 175	9.2 ± 0.9	6256 ± 261
1619	(SEQ ID No:38)	.........T.......	1555 ± 304	2908 ± 129	12.5 ± 1.0	8243 ± 698
1618	(SEQ ID No:7)	......A..T.......	2109 ± 291	2596 ± 166	12.9 ± 0.7	10425 ± 674
1639	(SEQ ID No:3)	.....AA..T.......	1827 ± 83	2012 ± 132	11.5 ± 0.4	9489 ± 103
1707	(SEQ ID No:39)	......A..TC......	ND	1147 ± 175	4.0 ± 0.2	3534 ± 217
1708	(SEQ ID No:40	.....CA..TG......	ND	59 ± 3	1.5 ± 0.1	466 ± 109

Dots indicate identity; CpG dinucleotides are underlined; ND= not done

[a]The experiment was done at least three times with similar results. The level of IL-6 of unstimulated control cultures of both CH12.LX and splenic B cells was ≤ 10 pg/ml. The IgM level of unstimulated culture was 547 ± 82 ng/ml. CpG dinucleotides are underlined and dots indicate identity. Cells were stimulated with 20 μM of various CpG O-ODN. Data present the mean ± SD of triplicates.

[b][^{3}H] Uridine uptake was indicated as a fold increase (SI: stimulation index) from unstimulated control (2322.67 ± 213.68 cpm).

[c]Measured by ELISA.

LPS-nonresponsive C2H/HeJ mouse produced similar levels of IL-6 in response to bacterial DNA. To analyze whether the IL-6 secretion induced by *E. coli* DNA was mediated by the unmethylated CpG dinucleotides in bacterial DNA, methylated *E. coli* DNA and a panel of synthetic ODN were examined. As shown in Table 3, CpG ODN significantly induced IL-6 secretion (ODN 5a, 5b, 5c) while CpG methylated *E. coli* DNA, or ODN containing methylated CpG (ODN 5f) or no CpG (ODN 5d) did not. Changes at sites other than CpG dinucleotides (ODN 5b) or methylation of other cytosines (ODN 5g) did not reduce the effect of CpG ODN. Methylation of a single CpG in an ODN with three CpGs resulted in a partial reduction in the stimulation (compare ODN 5c to 5e; Table 3).

Table 3. Induction of Murine IL-6 secretion by CpG motifs in bacterial DNA or oligonucleotides.

Treatment	IL-6 (pg/ml)
calf thymus DNA	≤10
calf thymus DNA + DNase	≤10
E. coli DNA	1169.5 ± 94.1
E. coli DNA + DNase	≤10
CpG methylated *E. coli* DNA	≤10
LPS	280.1 ± 17.1
Media (no DNA)	≤10
5a SEQ. ID. No:115 ATGGACTCTCCAGCGTTCTC	1096.4 ± 372.0
5b SEQ. ID. No:19 .....AGG....A.....	1124.5 ± 126.2
5c SEQ. ID. No:15 ..C.....G.....	1783.0 ± 189.5
5d SEQ. ID. No:114 .... AGG..C..T.....	≤10
5e SEQ. ID. No:116 ..C.....G..Z.....	851.1 ± 114.4
5f SEQ. ID. No:16 ..Z.....ZG..Z.....	≤10
5g SEQ. ID. No:18 ..C.....G.....Z..	1862.3 + 87.26

T cell depleted spleen cells from DBA/2 mice were stimulated with phosphodiester modified oligonucleotides (O-ODN) (20 μM), calf thymus DNA (50 μg/ml) or *E. coli* DNA (50 μg/ml) with or without enzyme treatment, or LPS (10 μg/ml) for 24 hr. Data represent the mean (pg/ml) ± SD of triplicates. CpG dinucleotides are underlined and dots indicate identity. Z indicates 5-methylcytosine.

1). IL-6 production plateaued at approximately 50 μg/ml of bacterial DNA or 40 μM of CpG O-ODN. The maximum levels of IL-6 induced by bacterial DNA and CpG ODN were 1-1.5 ng/ml and 2-4 ng/ml respectively. These levels were significantly greater than those seen-after stimulation by LPS (0.35 ng/ml) (Fig. 1A). To evaluate whether CpG ODN with a nuclease-resistant DNA backbone would also induce IL-6 production, S-ODN were added to T cell depleted murine spleen cells. CpG S-ODN also induced IL-6 production in a dose-dependent manner to approximately the same level as CpG O-ODN while non-CpG S-ODN failed to induce IL-6 (Fig. 1C). CpG S-ODN at a concentration of 0.05 μM could induce maximal IL-6 production in these cells. This result indicted that the nuclease-resistant DNA backbone modification retains the sequence specific ability of CpG DNA to induce IL-6 secretion and that CpG S-ODN are more than 80-fold more potent than CpG O-ODN in this assay system.

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Induction of Murine IL-6 murine by CpG DNA in vivo

To evaluate the ability of bacterial DNA and CpG S-ODN to induce Il-6 secretion *in vivo*, BALB/c mice were injected iv. with 100 μg of *E. coli* DNA, calf thymus DNA, or CpG or non-stimulatory S-ODN and bled 2 hr after stimulation. The level of IL-6 in the sera from the *E. coli* DNA injected group as approximately 13 ng/ml while IL-6 was not detected in the sera from calf thymus DNA or PBS injected groups (Table 4). CpG S-ODN also induced IL-6 secretion *in vivo*. The IL-6 level in the sera from CpG S-ODN injected groups was approximately 20 ng/ml. In contrast, IL-6 was not detected in the sera from non-stimulatory S-ODN stimulated group (Table 4).

Table 4. Secretion of Murine IL-6 induced by CpG DNA stimulation *in vivo*.

Stimulant	IL-6 (pg/ml)
PBS	< 50
E. coli DNA	13858 ± 3143
Calf Thymus DNA	< 50
CpG S-ODN	20715 ± 606
non-CpG S-ODN	< 50

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Mice (2 mice/group) were i.v. injected with 100 μl of PBS, 200 μl of *E. coli* DNA or calf thymus DNA, or 500 μg of CpG S-ODN or non-CpG control S-ODN. Mice were bled 2 hr after injection and 1:10 dilution of each serum was analyzed by IL-6 ELISA. Sensitivity limit of IL-6 ELISA was 5 pg/ml. Sequences of the CpG S-ODN is 5'GCATGACGTTGAGCT3' (SEQ. ID. No:6) and of the non-stimulatory S-ODN is 5'GCTAGATGTTAGCGT3' (SEQ. ID. No:4). Note that although there is a CpG in sequence 48, it is too close to the 3' end to effect stimulation, as explained herein. Data represent mean ± SD of duplicates. The experiment was done at least twice with similar results.

results are shown in Table 11.

Effective ODNs began with a TC or TG at the 5' end, however, this requirement was not mandatory. ODNs with internal CpG motifs (e.g., ODN 1840) are generally less potent stimulators than those in which a GTCGCT (SEQ. ID. NO: 58) motif immediately follows the 5' TC (e.g., ODN 1967 and 1968). ODN 1968, which has a second GTCGTT SEQ. ID. NO: 57) motif in its 3' half, was consistently more stimulatory than ODN 1967, which lacks this second motif. ODN 1967, however, was slightly more potent than ODN 1968 in experiments 1 and 3, but not in experiment 2. ODN 2005, which has a third GTCGTT (SEQ. ID. NO. 57) motif, inducing slightly higher NK activity on average than 1968. However, ODN 2006, in which the spacing between the GTCGTT (SEQ. ID. NO: 57) motifs was increased by the addition of two Ts between each motif, was superior to ODN 2005 and to ODN 2007, in which only one of the motifs had the additional of the spacing two Ts. The minimal acceptable spacing between CpG motifs is one nucleotide as long as the ODN has two pyrimidines (preferably T) at the 3' end (e.g., ODN 2015). Surprisingly, joining two GTCGTT (SEQ. ID. NO: 57) motifs end to end with a 5' T also created a reasonably strong inducer of NK activity (e.g., ODN 2016). The choice of thymine (T) separating consecutive CpG dinucleotides is not absolute, since ODN 2002 induced appreciable NK activation despite the fact that adenine (A) separated its CpGs (i.e., CGACGTT; SEQ. ID. NO: 113). It should also be noted that ODNs containing no CpG (e.g., ODN 1982), runs of CpGs, or CpGs in bad sequence contents (e.g., ODN 2010) had no stimulatory effect on NK activation.

Table 10

ODN	Sequence (5'-3')	LU	
cells alone		0.01	
1754	ACCATGGACGATCTGTTTCCCCTC	0.02	SEQ ID NO:59
1758	TCTCCCAGCGTGCGCCAT	0.05	SEQ ID NO:45
1761	TACCGCGTGCGACCCTCT	0.05	SEQ ID NO:60
1776	ACCATGGACGAACTGTTTCCCCTC	0.03	SEQ ID NO:61
1777	ACCATGGACGAGCTGTTTCCCCTC	0.05	SEQ ID NO:62
1778	ACCATGGACGACCTGTTTCCCCTC	0.01	SEQ ID NO:63
1779	ACCATGGACGTACTGTTTCCCCTC	0.02	SEQ ID NO:64
1780	ACCATGGACGGTCTGTTTCCCCTC	0.29	SEQ ID NO:65
1781	ACCATGGACGTTCTGTTTCCCCTC	0.38	SEQ ID NO:66
1823	GCATGACGTTGAGCT	0.08	SEQ ID NO:6
1824	CACGTTGAGGGGCAT	0.01	SEQ ID NO:67
1825	CTGCTGAGACTGGAG	0.01	SEQ ID NO:68
1828	TCAGCGTGCGCC	0.01	SEQ ID NO:69
1829	ATGACGTTCCTGACGTT	0.42	SEQ ID NO:70
1830	RANDOM SEQUENCE	0.25	
1834	TCTCCCAGCGGGCGCAT	0.00	SEQ ID NO:71
1836	TCTCCCAGCGCGCGCCAT	0.46	SEQ ID NO:72
1840	TCCATGTCGTTCCTGTCGTT	2.70	SEQ ID NO:73
1841	TCCATAGCGTTCCTAGCGTT	1.45	SEQ ID NO:74
1842	TCGTCGCTGTCTCCGCTTCTT	0.06	SEQ ID NO:75
1851	TCCTGACGTTCCTGACGTT	2.32	SEQ ID NO:76

results are shown in Table 11.

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1778	ACCATGGACGACCTGTTTCCCCTC	0.01	SEQ ID NO:63
1779	ACCATGGACGTACTGTTTCCCCTC	0.02	SEQ ID NO:64
1780	ACCATGGACGGTCTGTTTCCCCTC	0.29	SEQ ID NO:65
1781	ACCATGGACGTTCTGTTTCCCCTC	0.38	SEQ ID NO:66
1823	GCATGACGTTGAGCT	0.08	SEQ ID NO:6
1824	CACGTTGAGGGGCAT	0.01	SEQ ID NO:67
1825	CTGCTGAGACTGGAG	0.01	SEQ ID NO:68
1828	TCAGCGTGCGCC	0.01	SEQ ID NO:69
1829	ATGACGTTCCTGACGTT	0.42	SEQ ID NO:70
1830[2]	RANDOM SEQUENCE	0.25	

D'6 Cont'd

1834	TCTCCCAGCGGGCGCAT	0.00	SEQ ID NO:71
1836	TCTCCCAGCGCGCGCCAT	0.46	SEQ ID NO:72
1840	TCCATGTCGTTCCTGTCGTT	2.70	SEQ ID NO:73
1841	TCCATAGCGTTCCTAGCGTT	1.45	SEQ ID NO:74
1842	TCGTCGCTGTCTCCGCTTCTT	0.06	SEQ ID NO:75
1851	TCCTGACGTTCCTGACGTT	2.32	SEQ ID NO:76

Table 15. Specific blockade of CpG-induced TNF-α and IL-12 expression by inhibitors of endosomal acidification or NFκB activation

activators	Medium		Inhibitors: Bafilomycin (250 nM)		Chloroquine (2.5 μg/ml)		Monensin (10 μM)		NAC (50 mM)	TPCK (50 μM)	Glio-toxin (0.1 μg/ml)	Disglin-toxin (0.1 μg/ml)
	TNF-α	IL-12	TNF-α	IL-12	TNF-α	IL-12	TNF-α	IL-12	TNF-α	TNF-α	TNF-α	TNF-α
Medium	37	147	46	102	27	20	22	73	10	24	17	41
CpG ODN	455	17,114	71	116	28	6	49	777	54	23	31	441
LPS	901	22,485	1370	4051	1025	12418	491	4796	417	46	178	1120

Table 15 legend IL-12 and TNF-α assays: The murine monocyte cell line J774 ($1x10^5$ cells/ml for IL-12 or $1x10^6$ cells/ml for TNF-α), were cultured with or without the indicated inhibitors at the concentrations shown for 2 hr and then stimulated with the CpG oligodeoxynucleotide (ODN) 1826 (TCCATGACGTTCCTGACGTT SEQ ID NO:10) at 2μM or LPS (10 μg/ml) for 4 hr (TNF-α) or 24 hr (IL-12) at which time the supernatant was harvested. ELISA for IL-12 or TNF-α (pg/ml) was performed on the supernatants essentially as described (A.K. Krieg, A.-K. Yi, S. Matson, T.J. Waldschmidt, G.A. Bishop, R. Teasdale, G. Koretzky and D. Klinman, *Nature* 374, 546 (1995); Yi, A.-K., D.M. Klinman, T.L. Martin, S. Matson and A.M. Krieg, *J. Immunol.*, 157, 5394-5402 (1996); Krieg, A.M., *J. Lab. Clin. Med.*, 128, 128-133 (1996). Cells cultured with ODN that lacked CpG motifs did not induce cytokine secretion. Similar specific inhibition of CpG responses was seen with IL-6 assays, and in experiments using primary spleen cells or the B cell lines CH12.LX and WEHI-231.2.5μg/ml of chloroquine is equivalent to <5μM. Other inhibitors of NF-κB activation including PDTC and calpain inhibitors I and II gave similar results to the inhibitors shown. The results shown are representative of those obtained in ten different experiments.

Excessive immune activation by CpG motifs may contribute to the pathogenesis of the autoimmune disease systemic lupus erythematosus, which is associated with elevated levels of circulating hypomethylated CpG DNA. Chloroquine and related antimalarial compounds are effective therapeutic agents for the treatment of systemic lupus erythematosus and some other autoimmune diseases, although their mechanism of action has been obscure. Our demonstration of the ability of extremely low concentrations of chloroquine to specifically

Table 15. Specific blockade of CpG-induced TNF-α and IL-12 expression by inhibitors of endosomal acidification or NFκB activation

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	TNF-α	IL-12	TNF-α	IL-12	TNF-α	IL-12	TNF-α	IL-12	TNF-α	TNF-α	TNF-α	TNF-α
Medium	37	147	46	102	27	20	22	73	10	24	17	41
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D9 cont'd

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